

Cytokine Profile Associated with Human Chronic Schistosomiasis Mansonii

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This study objective was to evaluate the cytokines associated with early events of hepatic fibrosis in schistosomiasis mansonii. Hepatic fibrosis was classified by ultrasonography in 94 patients. Immunological evaluation was performed by measurement of secreted cytokines (interleukin IL-5, IL-10, IL-13, interferon- γ , tumor necrosis factor- α and transforming growth factors- β) in peripheral blood mononuclear cells stimulated by Schistosoma mansonii antigens. Significantly, higher levels of IL-5, IL-10 and IL-13 were found in supernatants of SEA-stimulated PBMC from subjects with degree III hepatic fibrosis as compared to patients with degree I or II fibrosis. Significant increases in IL-5 and IL-13 levels were also observed in some of the subjects who remained untreated for one year following initial assessment and developed more serious fibrosis during this period. The data suggests a role for type 2 cytokines in early stages of hepatic fibrosis in human schistosomiasis mansonii.

Key words: schistosomiasis - hepatic fibrosis - interleukin-5 - interleukin-13

The pathology resulting from infection with the helminth parasite *Schistosoma mansonii* is predominantly caused by the host immune response to parasite eggs that are laid in the portal venous system, and which become trapped in hepatic sinusoids and sequestered within granulomatous lesions (Warren 1968, Pessoa & Martins 1978, Cheever et al. 1994, Bica et al. 2000, Chiaromonte et al. 2001, Monica et al. 2003). The fibrosis associated with granuloma formation can lead to portal hypertension, which causes much of the morbidity and mortality associated with schistosomiasis (Doumenge 1987).

The formation of granulomas around schistosome eggs is mediated by CD4+ T cells (Warren et al. 1967). In the murine model of schistosomiasis, type 2 associated cytokines, including interleukin (IL-4, IL-5, IL-13) contribute to granuloma formation and the presence of eosinophils in these lesions (Chiaromonte et al. 2001). However, in human schistosomiasis, studies have shown that high levels of tumor necrosis factor- α and low levels of interferon- γ produced by in SEA stimulated peripheral blood mononuclear cells (PBMC) are significantly associated with the presence of severe liver fibrosis (Mwatha et al. 1998, Henri et al. 2002, Booth et al. 2004). As hepatosplenic

disease is a long-term complication of this schistosomiasis mansonii, it is conceivable that the immune mechanisms responsible for this lesion occur much earlier during infection and precedes the downstream development of hepatosplenomegaly. Consequently, it is important to evaluate the immune response in the early events of hepatic fibrosis. In the present study the cytokine profiles in supernatants of soluble egg Ag-stimulated PBMC from schistosomiasis patients with different stages of hepatic fibrosis were measured, and an association between type 2 cytokine production and progression to fibrosis was detected.

MATERIALS AND METHODS

Endemic area, subjects selected and ultrasonography - This study included 94 patients with schistosomiasis who had their degrees of fibrosis determined by ultrasonography. The majority of the patients (n = 79) were selected from Caatinga do Moura, at the village endemic for schistosomiasis mansonii, located at 380 km from Salvador, Bahia. A constant aquatic exposure is observed in a large part of the population. Because there were few patients with degree III hepatic fibrosis in Caatinga do Moura, more patients with degree III fibrosis were recruited from two other endemic areas of schistosomiasis: Taquarendi (n = 9) another village, located at 30 km from Caatinga do Moura, and Maruim (n = 6), a village from the state of Sergipe, north of the state of Bahia. All field sites are not endemic for malaria, leishmaniasis or Chagas disease but have other intestinal parasitic infections such as *Ascaris lumbricoides*, *Thichiuirus trichiura*, *Ancylostoma duodenale*, *Entamoeba coli*, *Entamoeba histolytica*, and *Giardia lamblia*. All patients with degree III of hepatic fibrosis had small spleen sizes (1 to 3 cm of the left costal

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ribs) and no past digestive bleeding or other signs of severe portal hypertension. The inclusion criteria were to have positive Kato-Katz parasitological examinations for schistosomiasis, no history of therapy for schistosomiasis in the last 3 years, and hepatic fibrosis as assessed by ultrasonography. Exclusion criteria included being under 4 or above 60 years or having positive serology for HIV (human immunodeficiency virus), HTLV-1 (human T cell leukemia virus type I) or hepatitis virus type B and C, as well as other conditions that could interfere in the immunological evaluation or ultrasonographical classification. Clinical and parasitological stool exams are performed in these populations every 3 years, followed by specific treatment for *S. mansoni* with oxaminiquine (20 mg/kg of weight). All patients or the guardians of minors signed an informed consent and the study was approved by the Ethical Committee of the Hospital Universitário Prof. Edgard Santos.

Ultrasonographical examination - Were performed using the WHO criteria established in 1993 for classification of hepatic fibrosis, (Abdel-Wahab et al. 1992, de Jesus et al. 2000). This classification considers degree 0, if periportal tract is < 3 mm; degree I, if it is from 3 to 5 mm; degree II, if it is from > 5 to 7 mm; and degree III, if it is > 7 mm. These exams were performed by two independent persons, and we have shown a correlation between periportal thickness and portal vein diameter and spleen size (de Jesus et al. 2000). Twenty-one of these subjects who failed to be treated, even after several attempts to localize them for schistosomicidal drugs, were re-evaluated by ultrasonographical exam after one year of the first evaluation. Nineteen of these patients had shown an increase in the periportal tract thickness, but in only 12 had this increase induced a change in their degrees of hepatic fibrosis. For 3 patients, this had changed from degree 0 to I and for 9 patients, this had changed from degree I to II. One year after treatment with oxaminiquine, all patients had improved and returned to degree 0 or I of fibrosis (de Jesus et al. 2000).

Immunological methods - PBMC were isolated from heparinized blood by density gradient centrifugation using histopaque 1077 (Sigma Diagnostic, St. Louis, MO) as previously described (Ribeiro de Jesus et al. 2000) and were left without stimulus or were stimulated with *S. mansoni* specific antigens: soluble extract of whole adult

S. mansoni SWAP (10 µg/ml) and soluble extract egg Ag, SEA (10 µg/ml), prepared as previously described (Pearce et al. 1986, Ribeiro de Jesus et al. 2000). The optimal concentration was determined previously by testing uninfected controls from Salvador. Levels of the cytokines (IL-5, IL-10, IFN-γ, TGF-β, IL-13, and TNF-α) were measured in supernatants using sandwich ELISAs, and the results were expressed as pg/ml, based on comparisons with standard curves, according to a previously described technique (Ribeiro de Jesus et al. 2000, 2002). Commercial kits from R&D (R&D systems Inc. Minneapolis, MN, US) were used for IFN-γ, IL-10, TNF-α, IL-13, and TGF-β. Duo set antibodies (PharMingen, San Diego, CA, US) were used to test IL-5. Immulon 2 ELISA plates were from Dynatech (Dynatech Laboratories, Inc., US). The sensitivity cut-offs for the ELISAs for each of the cytokines were: 30 pg/ml for IFN-γ, IL-5, IL-10, and TNF-α; 14 pg/ml for IL-13 and 7 pg/ml for transforming growth factor-β. The results considered for analysis were the differences of cytokine concentrations from the supernatants of stimulated culture versus unstimulated PBMC cultures.

Statistical methods - The differences in all variables analyzed between the groups of subjects with degrees I, II, and III of hepatic fibrosis were analyzed by the Mann-Whitney test. A non-parametric test was chosen because the data did not follow a Gaussian distribution. The differences in cytokine levels in the group of patients who increased the degree of fibrosis in 2 consecutive evaluations were compared by Wilcoxon signed rank test for matched groups. These statistical analyses were performed using the program InStat for Macintosh. An α value of 5% was considered for significance.

RESULTS

Description of the study groups - The 94 schistosomiasis patients selected for study had an age range of 5 to 55 with mean and standard deviation of 16 ± 8 , with 46 male and 48 females. Their infection levels ranged from 24 to 1784 e.p.g of feces. According to ultrasonographical examination, 8 subjects were classified as degree 0, 50 as degree I, 22 as degree II, and 14 as degree III of hepatic fibrosis. The age, sex, and infection levels in the subgroups of patients with different degrees of hepatic fibrosis are described in the Table. Patients with degree 0 and I of hepatic fibrosis were included in the same subgroup.

TABLE
Demographic data and infection intensity for schistosomiasis subjects with different degrees of hepatic fibrosis classified by ultrasonography

	Degrees of hepatic fibrosis		
	0 - I	II	III
Number of patients	58	22	14
Age range	5-55	9-45	7-50
Mean age ± standard deviation (SD)	16 ± 11.5^a	21 ± 10.5^a	21 ± 13.3
Sex (M:F)	31:27	11:11	8:6
Range of number of eggs/g stool	24-1784	40-1616	24-216
Mean ± SD number of eggs/g stool	379 ± 426^b	412 ± 399^b	$117 \pm 98^{b/c}$

a, b, c: p = 0.01

Differences in age were observed between patients with degrees 0-I (mean \pm SD of 16 ± 11.5) and II (21 ± 10.5) of hepatic fibrosis ($p = 0.01$, Mann-Whitney test). Differences in infection levels were also observed between the patients with degrees I and III (379 ± 426 and 117 ± 98 , respectively) and between degrees II and III (412 ± 399 and 117 ± 98 , respectively) of hepatic fibrosis ($p = 0.01$, Mann Whitney test).

The differences in the numbers of patients evaluated for each cytokine reflects the limited availability of culture supernatants, which itself reflects the limited numbers of PBMC obtained. Based on previous reports, we prioritized the evaluation of IFN- γ (SWAP $n = 94$, SEA $n = 62$) TNF- α (SWAP $n = 74$, SEA $n = 56$), and IL-5 (SWAP $n = 82$, SEA $n = 73$), leaving less supernatants for the measurement of IL-10 (SWAP $n = 74$, SEA $n = 54$), IL-13 (SEA $n = 39$), and TGF- β (SEA $n = 41$). Because patients were treated after donating PBMC samples, we were unable to perform repeat analyses. We were unable to assess IL-4 production due to the difficulty of measuring this cytokine in PBMC supernatants (Sabin et al. 1996).

Cytokine response to *S. mansoni* antigens - A great variability in cytokine levels was observed in general, showing the heterogeneity of the response to *S. mansoni* antigens. A significantly ($p = 0.03$, Mann Whitney test) higher level of IFN- γ was observed in supernatants of SWAP-stimulated PBMC from patients with degree II ($n = 23$) versus degree I ($n = 57$) versus degree III ($n = 14$) hepatic fibrosis (257 ± 354 versus 104 ± 189 vs 39 ± 58 pg/ml, respectively). No significant differences in IFN- γ levels were observed in supernatants from SEA stimulated PBMC from the three groups of patients (79 ± 295.8 , 3 ± 14.2 and 44 ± 79 pg/ml), (Fig. 1A). The levels of TNF- α in SWAP and SEA-stimulated PBMC supernatants also did not show any differences between the groups (Fig. 1B).

High levels of IL-5 were produced by SWAP-stimulated PBMC from all groups of patients (degree I 1043 ± 1844.5 , degree II 1319 ± 1706.7 , and degree III 2219 ± 2011), with significantly higher levels in patients with degree III versus degree I fibrosis ($p = 0.03$, Mann Whitney test). Levels of IL-5 were significantly lower in supernatants from SEA-stimulated PBMC of subjects with degrees I (93 ± 248.4 pg/ml) and II (11 ± 29.8 pg/ml) of hepatic fibrosis, than in supernatants from patients with degree III hepatic fibrosis (669 ± 815.6 pg/ml), ($p = 0.01$ and $p = 0.009$, respectively, Mann Whitney test) (Fig. 2A). IL-13 levels in supernatants of SEA stimulated PBMC were also higher in subjects with degree III fibrosis (116 ± 120.9 pg/ml), as compared to those with degrees I (24 ± 11.4 pg/ml) or II (86 ± 166.5 pg/ml) of hepatic fibrosis, ($p = 0.009$ and $p = 0.05$, respectively, Mann Whitney test) (Fig. 2B). Levels of IL-10 in SWAP-stimulated PBMC was not statistically significant. Higher levels of IL-10 were observed in supernatants of SEA-stimulated PBMC from patients with degree III hepatic fibrosis (187 ± 110 pg/ml) compared to those with degree I (63 ± 62.9 pg/ml) or II (66 ± 53 pg/ml) of hepatic fibrosis ($p = 0.01$ and $p = 0.03$, respectively, Mann Whitney test) (Fig. 3A). No differences were detected in TGF- β levels in SEA-stimulated PBMC supernatants between the groups of patients with degrees I, II, and III hepatic fibrosis. (Fig. 3B).

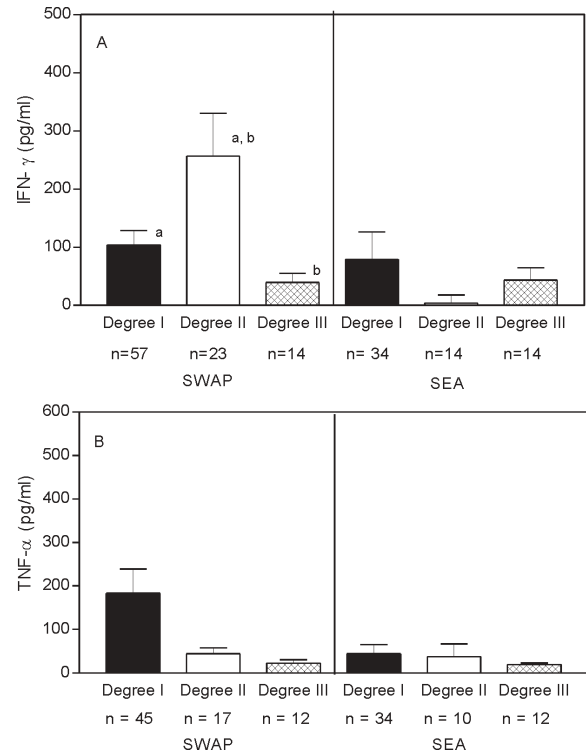


Fig.1: interferon- γ (A) and tumor necrosisfactor- α (B) levels in SWAP and SEA-stimulated peripheral blood mononuclear cells supernatants in schistosomiasis patients with different degrees of hepatic fibrosis. Data represents the mean \pm Standard Error (SEM). a: $p = 0.03$; b: $p = 0.02$, Mann Whitney test

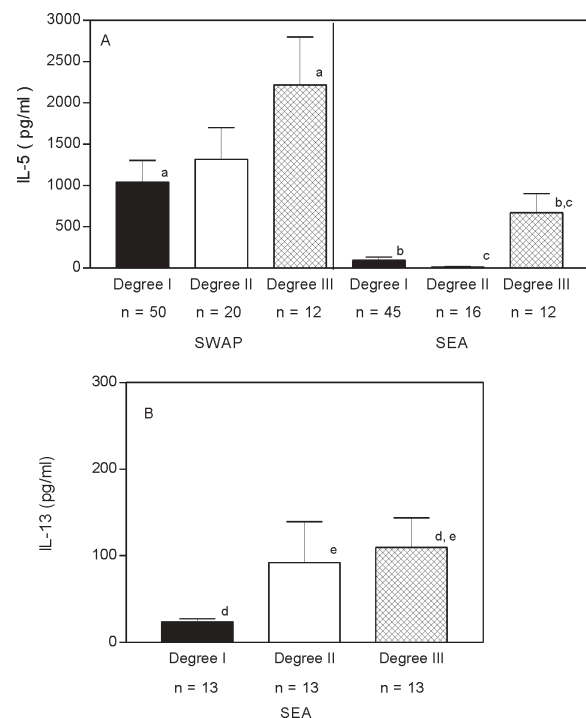


Fig. 2: interleukin-5 (A) and IL-13 (B) levels in SWAP and SEA-stimulated peripheral blood mononuclear cells supernatants in schistosomiasis patients with different degrees of hepatic fibrosis. Data represents the mean \pm Standard Error (SEM). a: $p = 0.03$; b: $p = 0.01$; c: $p = 0.009$; d: $p = 0.0009$; e: $p = 0.05$, Mann Whitney test

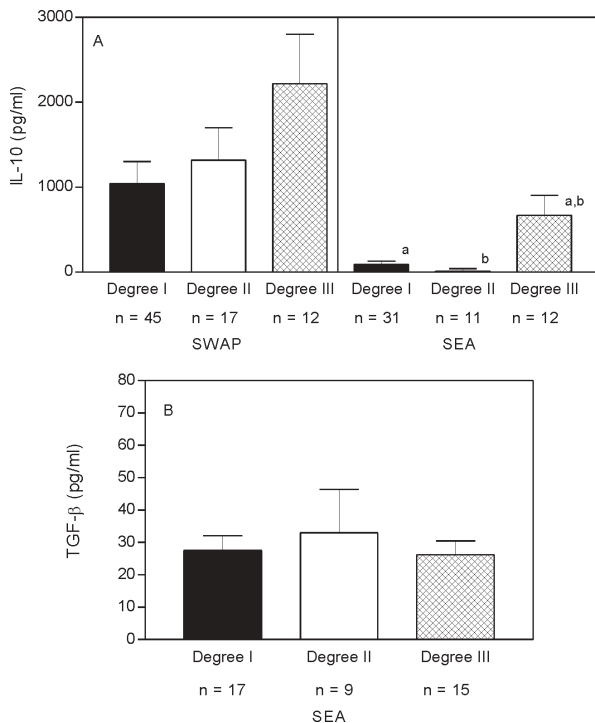


Fig. 3: interleukin-10 (A) and transforming growth factor- β (B) levels in SWAP and SEA-stimulated peripheral blood mononuclear cells supernatants in schistosomiasis patients with different degrees of hepatic fibrosis. Data represents the mean \pm Standard Error (SEM). a: $p = 0.01$; b: $p = 0.03$, Mann Whitney test

Responses of patients who remained untreated following initial assessment. Even after an active search, 21 patients missed treatment that should be performed at the end of the study, and remained untreated after one year from their first ultrasonography examination. Ultrasonography was repeated on these individuals and 19 (90%) of them were found to have increased periportal thickness and 2 of them remained with similar measurements. Within this group, 12/19 (57%) exhibited more severe fibrosis compared to the previous exam. In 3 patients the fibrosis index had changed from degree 0 to degree I, and in 9 patients the fibrosis index had increased from degree I to degree II. Immunological evaluation was repeated in these patients and compared with the first evaluation. An increase in the levels of some cytokines was observed in SEA-stimulated PBMC supernatants. Levels of IFN- γ were evaluated in 18 patients and 2 patient presented a decrease but the others did not show any significant change in the second versus first evaluations (Fig. 4A). TNF- α levels increased in 5 patients and decreased in 2 patients and remained at similar levels in 8 of the 15 patients tested (Fig. 4B). IL-5 levels increased in 9 patients, decreased in 1 patient and remained at similar levels in 8 of the 18 patients tested (Fig. 5A), and IL-13 levels showed a major increase in 5 patients, a minor increase in 2 patients and decreased or remained at similar levels in 2 of the 9 patients tested (Fig. 5B). The levels of IL-5 and IL-13 were significantly higher in the second evaluation (206 ± 434

and 89.7 ± 56 pg/ml, respectively), in comparison to the first evaluation (19 ± 62 pg/ml and 9.6 ± 11 pg/ml, respectively), ($p = 0.03$ and $p = 0.04$ respectively, Wilcoxon signed rank test). IL-10 levels also increased in 7 of 13 patients, decreased in 3 and remained at similar levels in other 3 patients (Fig. 6A), and TGF- β levels increased in 6 of 14 patients, decreased in 2 and remained at similar levels in other 2 patients (Fig. 6B). No statistically significant differences were observed in the levels of IFN- γ , TNF- α , IL-10 and TGF- β in SEA-stimulated PBMC supernatants from the second to the first evaluations ($p = 0.4$, $p = 0.4$, $p = 0.3$ and $p = 0.4$, respectively, Wilcoxon signed rank test). No significant differences were observed between the levels of cytokines in SWAP-stimulated PBMC supernatants from the second to the first evaluations.

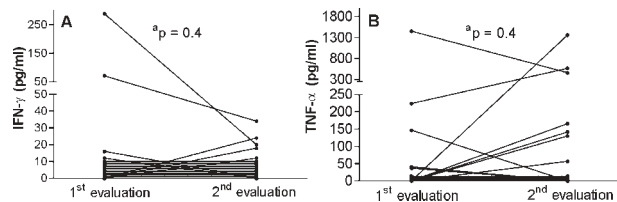


Fig. 4: interferon- γ (A) and tumor necrosis factor- α (B) levels in SEA-stimulated peripheral blood mononuclear cells supernatants from subjects who increased the periportal tract thickness after one-year follow-up without treatment. a: Wilcoxon signed rank test

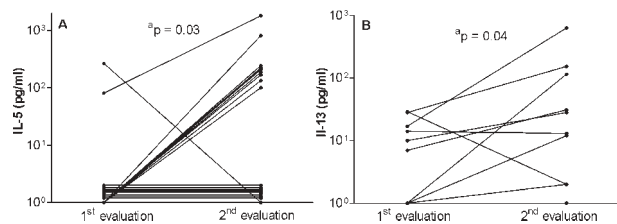


Fig. 5: interleukin-5 (A) and IL-13 (B) levels in SEA-stimulated peripheral blood mononuclear cells supernatants from subjects who increased the periportal tract thickness after one-year follow-up without treatment. IL-5 and IL-13 levels increased in (50%) of the patients. a: Wilcoxon signed rank test

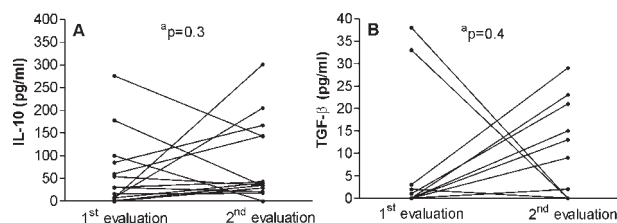


Fig. 6: interferon-10 (A) and transforming growth factor- β (B) levels in SEA-stimulated peripheral blood mononuclear cells supernatants from subjects who increased the periportal tract thickness after one-year follow-up without treatment. IL-10 levels increased in (39%), and TGF- β increased in (43%) of the patients. a: Wilcoxon signed rank test.

DISCUSSION

A type-2 cytokine pattern dominates the immune response in mice and humans chronically infected by *S. mansoni*. In mice, IL-4 and IL-13 play a major role in egg granuloma formation, and IL-13 was shown to play a significant role in the development of hepatic fibrosis (Chiaramonte et al. 1999, 2001, Monica et al. 2003). In humans, although low IFN- γ and high TNF- α are associated to severe hepatic fibrosis (Henri et al. 2002, Booth et al. 2004), it is still not clear which cytokines are involved in the progression of schistosomiasis mansoni pathology. The present study evaluated the cytokine profile in schistosomiasis patients developing hepatic fibrosis in pre-hepatosplenic and early hepatosplenic stages of the disease, and showed an association between a type 2 cytokine profile (the production of IL-5, IL-10, and IL-13) and degree III hepatic fibrosis. IL-5 and IL-13 showed the strongest association with severe hepatic fibrosis, and also increased significantly in patients who exhibited increased hepatic fibrosis after 1 year without treatment. Besides these Th2 cytokines, TGF- β also increased in about 50% of the patients who exhibit increased hepatic fibrosis after 1 year without treatment, remained at similar. However TGF- β levels did not differ in the groups with different degrees of hepatic fibrosis.

Many variables may influence the magnitude of the immune response in human schistosomiasis, including age, intensity of infection, and the type of Ag used to assess immune responsiveness. Moreover, increasing age is associated with an enhanced type 1 immune response (Ribeiro de Jesus et al. 1993) and heavy infections (more than 200 eggs/g stool) are associated with enhanced type 2 response (Araujo et al. 1996). These variables did not explain the results of the present study, since patients with degree III hepatic fibrosis made more IL-5 and IL-13 despite the fact that their age were higher than the other groups, and that they had the lowest intensity of infection.

Recent studies have pointed out an important role of IL-13 in the development of liver fibrosis (Chiaramonte et al. 2001, Monica et al. 2003). Although IL-4 and IL-13 share the same receptor and many biological activities, there are functional differences between the two cytokines. While granuloma formation was partially reduced in IL-4-deficient mice, blocking IL-13 and the IL-4 receptor in these animals almost completely abrogated granuloma development and tissue fibrosis (Chiaramonte et al. 2001). Our data, by showing a correlation between the production of high levels of IL-13 and the development of more severe fibrosis, suggests that this cytokine might also play a significant role in human schistosomiasis fibrosis. A recent study has found a protective effect of IFN- γ in liver fibrosis in Sudanese *S. mansoni*-infected subjects (Henri et al. 2002). Another recent study has also associated low IFN- γ production with liver fibrosis (Booth et al. 2004). In fact, earlier studies on schistosomiasis pathology in mice demonstrated that IFN- γ reduces the cellularity of granulomas, and downmodulates granuloma size (Boros & Lukacs 1992). These data are in concordance with the data from the present study, because IFN- γ is the major

type 1 cytokine involved in down modulate T helper type 2 cells.

In contrast to our data that showed increased IL-5 and IL-13 associated with liver fibrosis and that showed any significant associations between TNF- α levels and liver fibrosis, studies in schistosomiasis patients from Africa showed that patients with more severe hepatic fibrosis had higher TNF- α in PBMC supernatants (Mwatha et al. 1998, Henri et al. 2002, Booth et al. 2004). The contradictory results might be explained by the differences in the stages of the disease evaluated in these 2 studies: while the present study evaluated patients in the pre-hepatosplenic and early hepatosplenic stages of schistosomiasis mansoni, these studies evaluated hepatosplenic patients. Since the degree III hepatic fibrosis patients are in the early hepatosplenic phase of disease evolution, it is possible that the TNF- α is produced in later stages of the disease. Another possible explanation for the opposing results of these 2 studies is that the African population has a high prevalence of malaria, a protozoan infection that is known to cause hepatosplenomegaly and also to induce high levels of TNF- α (Mwatha et al. 1998, Clark 2003, Naus 2003). Further studies are necessary to better clarify the cytokines involved in advanced stages of hepatosplenic schistosomiasis, and the role of co-infections with malaria and other pathogens.

The documentation that IL-10 was found to be higher in subjects with degree III compared to degrees I and II hepatic fibrosis was unexpected. IL-10 is a modulatory cytokine which down regulates macrophage activation, MHC class I and II expression and reduces activation of both Th1 and Th2 cells (De Wall-Malefyt et al. 1993). Previous studies have shown that IL-10 is important to reduce pathology of acute schistosomiasis and it is decreased in patients with hepatosplenomegaly (Falcão et al. 1998). Since the patients in the present study were in the pre-hepatosplenic and early stages of hepatosplenomegaly it is possible that the documentation herein of increased levels of IL-10 in patients developing liver fibrosis may represent an attempt of this cytokine to down regulate the high levels of IL-5 and IL-13. The increment of TGF- β levels during evolution of hepatic lesions and lack of differences in subjects with degrees I, II, and III hepatic fibrosis could be due to an intermittent induction of fibrosis.

The present study presents evidence for a role of type 2 immune response in the development of liver fibrosis in human schistosomiasis. Since IL-5, IL-10, and IL-13 were associated with degree III hepatic fibrosis, and IL-5, IL-13 increased significantly in patients who developed more serious hepatic fibrosis over the course of the study.

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